Original Article

MicroRNA-19a inhibits nasopharyngeal carcinoma CNE1 cells viability and migration, but induces apoptosis by inhibition of Bcl-2

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Abstract: Nasopharyngeal carcinoma (NPC) is a highly metastatic cancer, frequently occurring in Southeast Asia and Southern China. Several microRNAs (miRNAs) have been shown to have an inhibitory effect on NPC, whereas the effect of miR-19a on NPC remains unclear. In this study, an effort has been made to study the potential effect of miR-19a on NPC cell viability, migration and apoptosis. Human NPC CNE1 cells were transfected with miR-19a mimic, miR-19a inhibitor, Bcl-2 targeted small interfering RNA (siRNA) and corresponding controls, respectively. RT-qPCR and western blot were performed to determine the expressions of miR-19a at mRNA and protein levels. Subsequently, cell viability, migration and apoptosis of the transfected cells were analyzed by Cell Counting Kit-8 (CCK-8), Transwell and flow cytometry assays. Analysis of signaling pathways were detected by western blot. Results showed that miR-19a overexpression inhibited cell viability and migration but promoted apoptosis in CNE1 cells ($P < 0.05$ or $P < 0.001$). Bcl-2 was negatively regulated by miR-19a, and Bcl-2 silence could dramatically induce apoptosis, and abolish miR-19a suppression induce increases in cell viability and migration ($P < 0.05$ or $P < 0.01$ or $P < 0.001$). Besides, Bcl-2 silence abolished miR-19a suppression induced activation of p38MAPK, p65 and IκBα. These results indicated that miR-19a inhibited cell viability, migration and induced apoptosis by down-regulating Bcl-2 in NPC cells.

Keywords: MicroRNA-19a, nasopharyngeal carcinoma, viability, migration, apoptosis, Bcl-2

Introduction

Nasopharyngeal carcinoma (NPC) is a rare malignant tumor deriving from epithelial cells located in the nasopharynx [1]. The incidence of NPC is higher in southern China and southeast Asia than in western countries [2]. Currently, the most effective treatment for NPC is radiotherapy, whereas the 5-year overall survival rate remains less than 50% [3]. It is a complex process of NPC occurrence, development, and metastasis. Growing evidence supports that microRNAs (miRNAs) play an important role in NPC [4]. Therefore, a better understanding of the molecular mechanisms of NPC will contribute to the development of new therapeutic and diagnostic strategies.

miRNAs are a family of small non-coding RNAs that range from 20-22 nucleotides in length [5]. An increasing number of studies have reported that miRNAs play important roles in the regulation of biological processes, such as cell differentiation, proliferation, migration and apoptosis [6]. Abnormal expressions in miRNAs are associated with cellular functions and also have been observed in a variety of diseases, including cancer [7]. Many miRNAs target oncogenes and tumor suppressor genes with direct involvement in carcinogenesis [8]. Currently, several miRNAs, such as miR-15a, miR-24, and miR-608 have been demonstrated to target specific mRNAs to regulate NPC development and progression [3]. Thus, it seems that modulation of miRNA could be a new approach for gene therapy of NPC [9]. MiR-19a belongs to miR-17-92 cluster, is associated with the pathogenesis and development of variety of human cancers, including cervical carcinoma [10], hepatocellular carcinoma [11], lung carcinoma [12] and prostate carcinoma [13]. However details of the function and molecular mechanism of miR-19a
in NPC cell proliferation, migration and apoptosis have not been well investigated.

Bcl-2 (B-cell lymphoma 2), encoded in humans by the BCL2 gene, is the founding member of the Bcl-2 family of regulator proteins that regulate cell apoptosis [14]. It is specifically considered an important anti-apoptotic protein but it is not considered a proto-oncogene because it is not a growth signal transducer [15]. Damage to the Bcl-2 gene has been identified as a cause of a number of cancers, including melanoma, breast cancer, prostate cancer, chronic lymphocytic leukemia and lung cancer [16]. Bcl-2 gene also has been reported as a possible cause of schizophrenia and autoimmunity [17]. Despite these observations, the Bcl-2 regulation in NPC, particularly through miRNAs remains unclear.

In the present study, we intended to find out the molecular mechanism of miR-19a in NPC cells, as well as to find out whether miR-19a plays deleterious or protective role in NPC by regulating Bcl-2. Human NPC cell line CNE1 was transfected with miR-19 mimic, miR-19 inhibitor, Bcl-2 targeted small interfering RNA (siRNA) and their controls. Then cell viability, migration and apoptosis were determined by Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, the cells were cultivated in 96-well plates and transfected with miR-19a mimic, miR-19a inhibitor, Bcl-2 siRNA, mimic control and inhibitor control. Forty-eight hours later, 10 μl of CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Migration assay

Cell migration was determined by using a modified two-chamber migration assay with a pore size of 8 mm. In brief, cells suspended in 200 ml of serum-free medium were seeded on the upper compartment of 24-well transwell culture chamber, and 600 ml of complete medium was added to the lower compartment. After a 12 h-incubation at 37°C, cells were fixed with 4% methanol (NIST, USA) for 30 min. Nontraversed cells were removed from the upper surface of the filter carefully with a cotton swab. Cells that migrated to the lower side of the membrane were stained with 0.1% crystal violet (Merck, Darmstadt, Germany) for 20 min, and images were captured using an inverted microscope (Olympus, Tokyo, Japan) [19].

Materials and methods

Cell culture

The Hubei Cancer Hospital (Wuhan, China) kindly provided the human NPC cell line CNE1 for the present study. CNE1 cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, Logan City, Utah, USA) with 10% fetal bovine serum (FBS) (HyClone) at 37°C with 5% CO2 [18].

Cell transfection

The mimic control, miR-19a mimic, inhibitor control, miR-19a inhibitor and Bcl-2 targeted siRNA were synthesized by Genepharma (Shanghai, China). CNE1 cells were seeded in 6-well plates and cultured overnight at 37°C in a humidified atmosphere of 95% air and 5% CO2. Subsequently, transfections were performed by using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell viability

Cells were seeded in 96-well plate with 5000 cells/well for adherence, and then cell viability was assessed by a Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Gaithersburg, MD). Briefly, the cells were cultivated in 96-well plates and transfected with miR-19a mimic, miR-19a inhibitor, Bcl-2 siRNA, mimic control and inhibitor control. Forty-eight hours later, 10 μl of CCK-8 solution was added to the culture medium, and the cultures were incubated for 1 h at 37°C in humidified 95% air and 5% CO2. The absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA).

Apoptosis assay

Cell apoptosis was performed by using the FITC Annexin V Apoptosis Detection Kit (Beijing Biosea Biotechnology, Beijing, China). Briefly, miR-transfected cells were washed twice in PBS and stained in 5 μl PI and 10 μl FITC-Annexin V in the presence of 50 μg/ml RNase A (Sigma-Aldrich), and then incubated for 1 h at room temperature in the dark. Flow cytometry analysis was done by using a FACS can (Beckman Coulter, Fullerton, CA, USA).
RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNAs of CNE1 cells were extracted from transfected cells by Trizol Reagent (Invitrogen). For miR-19a detection, reverse-transcribed complementary DNA (cDNA) was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Dalian, China), and RT-qPCR was performed with SYBR Premix ExTaq (TaKaRa) with the Stratagene Mx3000P real-time PCR system (Agilent Technologies, Inc., Santa Clara, CA, USA). Expression levels were normalized against the endogenous snRNA U6 control. For mRNA analyses, cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). RT-qPCR was performed with SYBR Premix ExTaq with the Stratagene Mx3000P real-time PCR system. GAPDH was used as internal controls for mRNA quantification. The relative expression ratio of mRNA was calculated by the $2^{-\Delta\Delta CT}$ method. PCR reactions for each gene were repeated three times. Independent experiments were done in triplicate.

Western blot

The protein used for western blot was extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Basle, Switzerland). The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amounts of protein were subjected to 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels, and then were transferred onto polyvinylidene fluoride (PVDF) membranes (Maibio, Shanghai, China). The transfer membranes were then incubated with 5% skim milk (Nestlé, Shuangcheng, China) in Tris Buffered Saline with Tween (TBST) for 2 h and probed with the following primary antibodies overnight at 4°C at a dilution of 1:1,000: BCL2-Associated X (Bax, ab32503), Bcl-2 (ab32124), pro-caspase-3 (ab32150), cleaved caspase-3 (ab13585), pro-caspase-9 (ab32068), phosphorylated (p)-p38MAPK (ab4822), p38MAPK (ab31828), p-p65 (ab86299), p65 (ab16502), p-IκBα (ab32518), IκBα (ab92700), GAPDH (ab8245, Abcam, Cambridge, UK) and cleaved caspase-9 (#9501; Cell signaling Technology). Thereafter, the membranes were incubated with the membrane at 4°C overnight, followed by incubation with secondary antibody (1:5000, Abcam, USA) marked by horseradish peroxidase for 1 h at room temperature. After rinsing, the PVDF membrane carried blots and antibodies were transferred into the Bio-Rad ChemiDoc™ XRS system, and then 200 µl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, US) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad).

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as the mean ± SD. Statistical analyses were performed using Graphpad 6.0 statistical software (GraphPad Software, San Diego, CA), and one-way analysis of variance (ANOVA). A $P$-value of < 0.05 was considered to indicate statistically significant results.

Results

**MiR-19a inhibited cell viability and migration, but promoted apoptosis of CNE1 cells**

In order to determine whether miR-19a was involved in the regulation of human NPC tumorigenesis, miR-19a mimic, miR-19a inhibitor, and
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their corresponding controls were respectively transfected into CNE1 cells. Transfection efficiency was examined by RT-qPCR, and results showed that miR-19a expression was significantly up-regulated by miR-19a mimic ($P < 0.01$) and down-regulated by miR-19 inhibitor ($P < 0.01$; Figure 1).

Next, CCK-8, Transwell and flow cytometry assays were conducted to investigate the biological functions of miR-19a on CNE1 cells. As results shown in Figure 2A and 2B, cell viability and migration rates in miR-19a-overexpressing cells were much lower than those in mimic control ($P < 0.05$), while these two rates in miR-19a-suppressing cells were higher than in inhibitor control ($P < 0.05$). Results in Figure 2C showed an increase of apoptotic cells percentage in miR-19a-overexpressing cells compared to mimic control ($P < 0.001$). However, miR-19a suppression had no obvious impact on cell apoptosis. In addition, western blot analysis was performed to detect the protein expression levels of apoptosis-related factors. Results showed that miR-19a overexpression up-regulated Bax expression and activated cleaved caspase-3 and cleaved caspase-9 expressions, while miR-19a suppression showed an opposite impact on these factors expressions. The protein levels of pro-caspase-3 and pro-caspase-9 were not significantly regulated by miR-19a overexpression or suppression (Figure 2D). These results indicated that miR-19a might be act as a tumor suppressor gene by inhibiting NPC cells viability and migration, as well as improving cell apoptosis.

Figure 2. MiR-19a inhibited cell viability and migration, but induced apoptosis in CNE1 cells. MiR-19a mimic, miR-19a inhibitor or controls were transfected into CNE1 cells, and then (A) cell viability, (B) migration, (C) apoptosis, and (D) the protein expressions of apoptosis-related factors were respectively detected by CCK-8, Transwell, flow cytometry, and western blot assays. MiR-19a: microRNA-19a; CCK-8: Cell Counting Kit-8; NPC: nasopharyngeal carcinoma; *$P < 0.05$, ***$P < 0.001$. 

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To further explore the effect of miR-19a on the regulation of NPC cells, miR-19a mimic, miR-19a inhibitor and their corresponding controls were transfected into CNE1 cells, and the mRNA and protein levels of Bcl-2 were detected by RT-qPCR and western blot. Results showed that the expression of Bcl-2 was significantly down-regulated in miR-19a-overexpressing cells, while was obviously up-regulated in miR-19a-suppressing cells (\(P < 0.05\), \(P < 0.01\)) (Figure 3A and 3B). Thus, we inferred that Bcl-2 was negatively regulated by miR-19a.

Bcl-2 was negatively regulated by miR-19a

To further explore the effect of miR-19a on the regulation of NPC cells, miR-19a mimic, miR-19a inhibitor and their corresponding controls were transfected into CNE1 cells, and the mRNA and protein levels of Bcl-2 were detected by RT-qPCR and western blot. Results showed that the expression of Bcl-2 was significantly down-regulated in miR-19a-overexpressing cells, while was obviously up-regulated in miR-19a-suppressing cells (\(P < 0.05\), \(P < 0.01\)) (Figure 3A and 3B). Thus, we inferred that Bcl-2 was negatively regulated by miR-19a.

**Bcl-2 was negatively regulated by miR-19a**

MAPks and NF-kB pathways have been reported to involve in different biological and pathological processes [20]. In our study, we analyzed the MAPK and NF-kB pathways by detection the expressions of p38MARK, p65 and IκBα. Western blot results showed that the expression of p-p38MAPK, p-p65 and p-IκBα were significantly promoted by miR-19a suppression, whereas these activations were remarkably abolished by Bcl-2 silence (Figure 5). Results indicated that miR-19a regulated the expression of Bcl-2 possibly via MAPK and NF-kB signaling pathways, and further regulated cell viability, migration and apoptosis.

**Discussion**

More and more evidences indicated that miRNAs might contribute to the pathogenesis of tumor, and their functions and potential application have become hot research areas [21]. To date, approximately 32 miRNAs were studied in NPC [22], whereas the effect of miR-19a on NPC remains unclear. In the present study, we demonstrated that overexpression of miR-19a inhibited CNE1 cells viability and migration, but promoted apoptosis. In addition, the suppressive effect of miR-19a in CNE1 cells possibly via inhibition of Bcl-2. Further, miR-19a suppression promoted the activations of p38MAPK, p65 and IκBα, while these activations were remarkably abolished by Bcl-2 silence.
Aberrant expression of miR-19a have been reported frequently in cancers, such as lung cancer [23], gastric cancer [24], thyroid cancer and cervical cancer [25]. In addition, miR-19a regulated key genes related to cell viability, migration and apoptosis. Specifically, miR-19a was up-regulated in lung cancer and exhibited promoting effects on cell proliferation and migration [23]. Besides, up-regulation of miR-19a enhanced cell proliferation and augmented the tumorigenesis of HepG2 cells in vitro [26]. Similarly, results of our study showed that overexpression of miR-19a inhibited cell viability and migration, but promoted cell apoptosis in CNE1 cells. We inferred that miR-19a might act as a tumor suppressor of NPC.

Bcl-2 gene is originally identified at a breakpoint of translocations commonly occurring in follicular B cell lymphomas and was later found to play a key role in cell survival and inhibition of apoptosis [27, 28]. Many studies have already showed that Bcl-2 could be regulated by several miRNAs [29]. For instance, Bcl-2 has been reported as a direct target of miR-15 and miR-16 in chronic lymphocytic leukemia [30]. In
addition, Su et al. reported that Bcl-2 was negatively regulated by miR-429 in NPC cells [31]. However, there was not adequate information to prove the effect of Bcl-2 via regulating miR-19a on NPC. Thus, our study for the first time uncovered the underlying relationship between miR-19a and Bcl-2 in NPC. These results demonstrated that Bcl-2 was negatively regulated by miR-19a in NPC. Moreover, Bcl-2 silencing could abolish the promoting effect of miR-19a suppression on cell viability and migration, and could promote apoptosis. Thus it could be seen that Bcl-2 plays a key role in cell viability, migration and apoptosis through regulation of miR-19a.

MAPKs (including ERK, JNK and p38) are serine-threonine kinases that regulate intracellular signaling associated with several cellular activities, such as cell proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis [32]. The transcription factors of NF-κB family are common and pleiotropic molecules that regulate the expression of more than 150 genes involved in a wide range of processes, including inflammation, immunity, cell proliferation, differentiation, and survival [33]. Many studies have established that MAPKs and NF-κB pathways play important roles in different biological and pathological processes [34]. Kutlu et al. found that up-regulation of miR-1182 activated MAPK signal pathway, thereby inducing melanoma cells apoptosis [35]. Moreover, Kim et al. demonstrated that miR-125a/b induced diffuse large B-cell lymphoma apoptosis via activating NF-κB signal pathway [36]. However, to our knowledge, none of studies have reported the effect of Bcl-2 on MAPK and NF-κB signaling pathways by regulation of miR-19a. Herein, our study analyzed the activation of MAPK and NF-κB pathways by the expression of p38, p65 and IκBα. These results suggested that miR-19a suppression promoted the activations of p38MAPK, p65 and IκBα, while these activations were remarkably abolished by Bcl-2 silence which indicating miR-19a regulated the expression of Bcl-2 via MAPK and NF-κB pathways.

To sum up, this study demonstrated that miR-19a inhibited cell viability and migration, but induced apoptosis in CNE1 cells, which indicating miR-19a may be a tumor suppressor gene of NPC. Besides, the tumor suppressive effects of miR-19a on CNE1 cells possibly via inhibition of Bcl-2, and inactivation of MAPK and IκBα pathways. These results provides basic information for the potential usages of miR-19a in treating NPC, but further studies on the regulatory mechanisms remain necessary.

Disclosure of conflict of interest
None.

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